Detection of Glutathione in Oral Squamous Cell Carcinoma Cells With a Fluorescent Probe During the Course of Oxidative Stress and Apoptosis

Yu Zhou, MD, Shengyun Huang, MD,† Hongzhou Shen, PhD,‡ Meng Ma, PhD,§ Baocun Zhu, MD,‖ and Dongsheng Zhang, MD¶*

Purpose: To detect glutathione (GSH) in oral squamous carcinoma cells (OSCCs) with a GSH selective fluorescent probe during the course of oxidative stress and apoptosis.

Materials and Methods: A novel GSH probe was applied to assess GSH in human tongue squamous cell carcinoma cells (cal-27). The cellular GSH and reactive oxygen species (ROS) levels were assessed with a GSH probe and DCF-DA (2,7-dichlorofluorescin diacetate) probe. The mitochondrial GSH and ROS levels were assessed with a GSH probe, DCF-DA probe, and Mitotracker Red CM-H_2XRos probe (Invitrogen, Carlsbad, CA). To further study whether oxidative stress would induce apoptosis of OSCCs, we then applied a GSH probe and annexin V–fluorescein isothiocyanate probe to assess cellular GSH levels and eversion of phosphatidylserine, and the cellular GSH levels and mitochondrial membrane potential ($\Delta\Psi m$) were assessed with a GSH probe and JC-1 probe during the course of oxidative stress and apoptosis induced by hydrogen peroxide and ethacrynic acid. The fluorescence was observed under laser confocal fluorescence microscopy.

Results: The intensity of fluorescence that represented intracellular alteration of GSH levels, cellular ROS formation, mitochondrial ROS formation, and apoptosis occurrence, respectively, could be visualized under laser confocal fluorescence microscopy.

Conclusions: The GSH selective fluorescent probe can evaluate cellular GSH levels sensitively during the course of oxidative stress and apoptosis of OSCCs induced by exogenous hydrogen peroxide, which could be enhanced by depletion of mitochondrial GSH.

J Oral Maxillofac Surg 75:223.e1-223.e10, 2017

*Resident, Department of Oral and Maxillofacial Surgery, School of Stomatology, Shandong University, Jinan, China; Department of Oral and Maxillofacial Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China; Department of Oral and Maxillofacial Surgery, Anhui Provincial Hospital Affiliated to Anhui Medical University, Hefei, China.

†Resident, Department of Oral and Maxillofacial Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China.

‡Resident, Department of Oral and Maxillofacial Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China.

§Resident, Department of Oral and Maxillofacial Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China.

‖Professor, School of Resources and Environment, University of Jinan, Jinan, China.

¶Professor and Department Head, Department of Oral and Maxillofacial Surgery, Shandong Provincial Hospital Affiliated to Shandong University; Department of Oral and Maxillofacial Surgery, School of Stomatology, Shandong University, Jinan, China.

Financial support was provided by Shandong Provincial Natural Science Foundation (CN) (grant No. ZR2012HQ022).

Address correspondence and reprint requests to Dr Zhang: Department of Oral and Maxillofacial Surgery, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jingwu Road, Jinan 250021, China; e-mail: ds63zhang@sdu.edu.cn

Received May 26 2016

Accepted August 8 2016

0278-2391/16/30740-6

http://dx.doi.org/10.1016/j.joms.2016.08.010

Oral squamous cell carcinoma is the most common head and neck malignant neoplasm, with high recurrence and metastasis rates. Chemotherapy is one of the main therapeutic strategies and suffers more and more resistance.[1] Reactive oxygen species (ROS) are the most plentiful free radicals with unpaired electrons in mammalian cells.[2] ROS formation is the fundamental instrument of chemotherapy agents and ionizing radiation, based on the rationale that persistent oxidative stress can cause the collapse of the intracellular antioxidant system and apoptosis of tumor cells.[3,4] The intracellular oxidation-reduction (redox) state remains homeostasis, which is tightly regulated by intracellular antioxidant systems.[5] Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) is the most abundant non-protein thiol and redox buffer in mammalian cells to provide the first-line defense of ROS.[6,7] GSH can not only directly interact with ROS or act as a substrate for different enzymes to eliminate endogenous and exogenous compounds, but also conjugate with xenobiotics such as chemotherapy agents directly.[8-10] So there is an intimate relationship between high GSH levels and anticancer drug resistance.[11,12]

Most ROS are generated from the mitochondrial respiratory chain.[3] Mitochondrial DNA, lipids, and proteins are susceptible to be damaged by oxidative stress because they are closer to the region where ROS are produced.[13] Therefore, mitochondria play a considerable role in apoptosis induced by oxidative stress, which is executed through an external or internal pathway and depends on mitochondrial dysfunction.[14,15] ROS can activate apoptosis through a mitochondrial pathway by opening of the mitochondrial permeability transition pore.[8] GSH is synthesized in cytoplasm and distributed into intracellular organelles mainly including mitochondria, nucleus, and endoplasmic reticulum.[9] Mitochondrial glutathione (mtGSH) plays a pivotal role in retaining mitochondrial redox homeostasis and helping cells escape apoptosis.[12,13] Oxidative damage to mitochondria can be prevented by mtGSH chiefly through the role of mitochondrial S-transferases (GSH S-transferase).[13] Thus, mitochondrial dysfunction, mitochondrial permeability transition pore opening and subsequent apoptotic cascade all result from inhibition of mtGSH.[16,17]

During the past several years, a few fluorescent probes have been reported for quantitative detection of GSH.[18] We have already devised a colorimetric and ratiometric fluorescent probe to detect GSH in living HeLa cells.[19] In this research, the GSH selective probe was applied to investigate the relationship between depletion of GSH and apoptosis of oral squamous carcinoma cells (OSCCs). Hydrogen peroxide (H_2O_2), the representative of ROS, is always applied to model oxidative stress.[20] Ethacrynic acid (EA), an effective inhibitor of GSH S-transferase P1-1, can deplete more mtGSH than cytoplasmic GSH and has been proved to enhance the toxic effect of chemotherapeutic agents such as cisplatin.[17,21-23] Variation of fluorescence intensity that represented intracellular alteration of GSH levels, cellular ROS formation, mitochondrial ROS formation, and apoptosis occurrence, respectively, could be visualized under laser confocal fluorescence microscopy. The level of GSH and cellular and mitochondrial ROS formation, as well as apoptosis occurrence, could be evaluated by observing the variation of fluorescence intensity under laser confocal fluorescence microscopy.

The purpose of this study was to determine whether the GSH selective fluorescent probe could be applied to detect alteration of cellular GSH levels sensitively during the course of oxidative stress and apoptosis of OSCCs induced by exogenous H_2O_2, as well as enhanced by depletion of mtGSH. The intact process of oxidative stress and apoptosis of OSCCs by depletion of GSH and mtGSH could be visualized. It proved that depletion of cellular GSH and mtGSH would facilitate apoptosis during the course of oxidative stress through the study, which showed a promising way to avoid resistance to therapy by monitoring GSH levels of patients who received chemotherapy or radiotherapy with the probe.

Materials and Methods

REAGENTS

The GSH fluorescent probe was provided by Professor Baocun Zhu (School of Resources and Environment, University of Jinan, Jinan, China). The following products were used for this study: Dulbecco's modified eagle medium high-glucose culture medium, fetal bovine serum, streptomycin-penicillin, and 0.25% trypsin (HyClone; GE Healthcare, Buckinghamshire, UK); *N*-acetyl-L-cysteine (NAC), H_2O_2, EA, and dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO); Mitotracker Red CM-H_2XRos (Invitrogen, Carlsbad, CA); ROS Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); and mitochondrial membrane potential assay kit with JC-1 and annexin V–fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BIOBOX, Nanjing, China).

CELL CULTURE

Human tongue squamous cell carcinoma cell lines (cal-27) were provided by Professor Wantao Cheng

(The Ninth People's Hospital Affiliated to Shanghai Jiao Tong University, Shanghai, China). The cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 1% streptomycin-penicillin and were maintained in an incubator containing a 5% carbon dioxide/air environment at 37°C. The cells were cultured in 25-cm^2 culture flasks and harvested in a logarithmic phase of growth with 0.25% trypsin.

APPLICATION OF GSH PROBE TO ASSESS GSH LEVELS

First, we applied the GSH probe to detect alteration of GSH levels in the cal-27 cells during the course of oxidative stress induced by exogenous H_2O_2 and depletion of mtGSH. In brief, 1×10^4 cal-27 cells were seeded in a 15-mm glass-bottom cell culture dish (NEST Biotechnology, Hong Kong, China) and cultured for 24 hours under the same conditions described earlier. The cells were then incubated with the GSH fluorescent probe (10 μmol/L) for 20 minutes at room temperature (20°C) and treated with 100-μmol/L H_2O_2 for 30 minutes. The cells were washed gently with phosphate-buffered saline solution (PBS) twice and treated with 1-mmol/L NAC for 1 hour. Finally, after the cells were washed gently with PBS twice, 50-μmol/L EA was added to the cells for 30 minutes. Blue fluorescence, which represented cellular GSH levels, was observed under a laser confocal fluorescence microscope at an excitation wavelength of 340 nm, and the observed field of the microscope remained the same.

ASSESSMENT OF INTRACELLULAR ROS AND GSH LEVELS

ROS levels were assessed using the ROS Assay Kit. In brief, 1×10^4 cal-27 cells were harvested and cultured under the same conditions described earlier. The cells were then incubated with DCF-DA (2,7-dichlorofluorescin diacetate) (10 μmol/L) for 30 minutes at room temperature (20°C) to detect ROS and with the GSH probe (10 μmol/L) for the last 20 minutes at room temperature (20°C) to detect GSH. Following the manufacturer's instructions, green fluorescence and blue fluorescence, which represented ROS and GSH levels, were observed under the laser confocal microscope at excitation wavelengths of 500 and 340 nm, respectively. After the cells were treated with 100-μmol/L H_2O_2 for 30 minutes, alteration of the fluorescence was imaged. Then the cells were washed gently with PBS twice and treated with 50-μmol/L EA for another 30 minutes.

ASSESSMENT OF MITOCHONDRIAL ROS AND GSH LEVELS

Next, we further detected mitochondrial ROS formation after oxidative damage by exogenous H_2O_2 and depletion of mtGSH by EA. We applied Mitotracker Red CM-H_2XRos to locate mitochondria. In brief, 1×10^4 cal-27 cells were harvested and cultured in a 15-mm glass-bottom cell culture dish for 24 hours under the same culture conditions described earlier. The cells were then incubated with DCF-DA (10 μmol/L) for 30 minutes at room temperature (20°C) to detect ROS, with the GSH fluorescent probe (10 μmol/L) for 20 minutes at room temperature (20°C) to detect GSH, and with Mitotracker Red CM-H_2XRos (200 nmol/L) to stain mitochondria for 20 minutes at room temperature (20°C). Green, blue, and red fluorescence was observed under the confocal microscope at excitation wavelengths of 500, 340, and 579 nm, respectively, following the manufacturer's instructions. Subsequently, the cells were treated as described earlier.

ANNEXIN V–FITC STAINING FOR APOPTOSIS AND ASSESSMENT OF GSH LEVELS

We used the annexin V–FITC probe to detect eversion of phosphatidylserine, which meant pristine apoptosis. In brief, 1×10^4 cal-27 cells were harvested and cultured in a 15-mm glass-bottom cell culture dish for 24 hours under the same culture conditions described earlier. The cells were then incubated with the GSH fluorescent probe (10 μmol/L) for 20 minutes at room temperature (20°C) to detect GSH and with the annexin V–FITC probe (5 μL of annexin V–FITC plus 500 μL of binding liquid) for the last 10 minutes at room temperature (20°C) to detect eversion of phosphatidylserine. Following the manufacturer's instructions, blue fluorescence, which meant GSH levels, and green fluorescence, which meant eversion of phosphatidylserine, were observed under the laser confocal fluorescence microscope at excitation wavelengths of 340 and 488 nm, respectively. Subsequently, the cells were treated as described earlier and alteration of fluorescence was imaged.

ASSESSMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL (ΔΨM) AND GSH LEVELS

We used a JC-1 probe to detect loss of mitochondrial transmembrane potential. In brief, the cal-27 cells were harvested and cultured in a 15-mm glass-bottom cell culture dish for 24 hours under the same culture conditions described earlier. The cells were washed twice with 1x JC-1 binding liquid and incubated with 500 μL of JC-1 working solution

at room temperature (20°C) for 20 minutes; then the cells were washed gently with PBS twice and incubated with 10-μmol/L GSH probe at room temperature (20°C) for another 20 minutes. Following the manufacturer's instructions, red fluorescence, which represented JC-1 polymer, and blue fluorescence, which represented GSH levels, were observed under the laser confocal fluorescence microscope at excitation wavelengths of 535 and 340 nm, respectively. Subsequently, the cells were treated as described earlier and alteration of fluorescence was imaged.

Results

ASSESSMENT OF CELLULAR GSH QUANTITATIVELY IN CAL-27 CELLS

After the cal-27 cells were incubated with the GSH probe for 20 minutes, intensive blue fluorescence

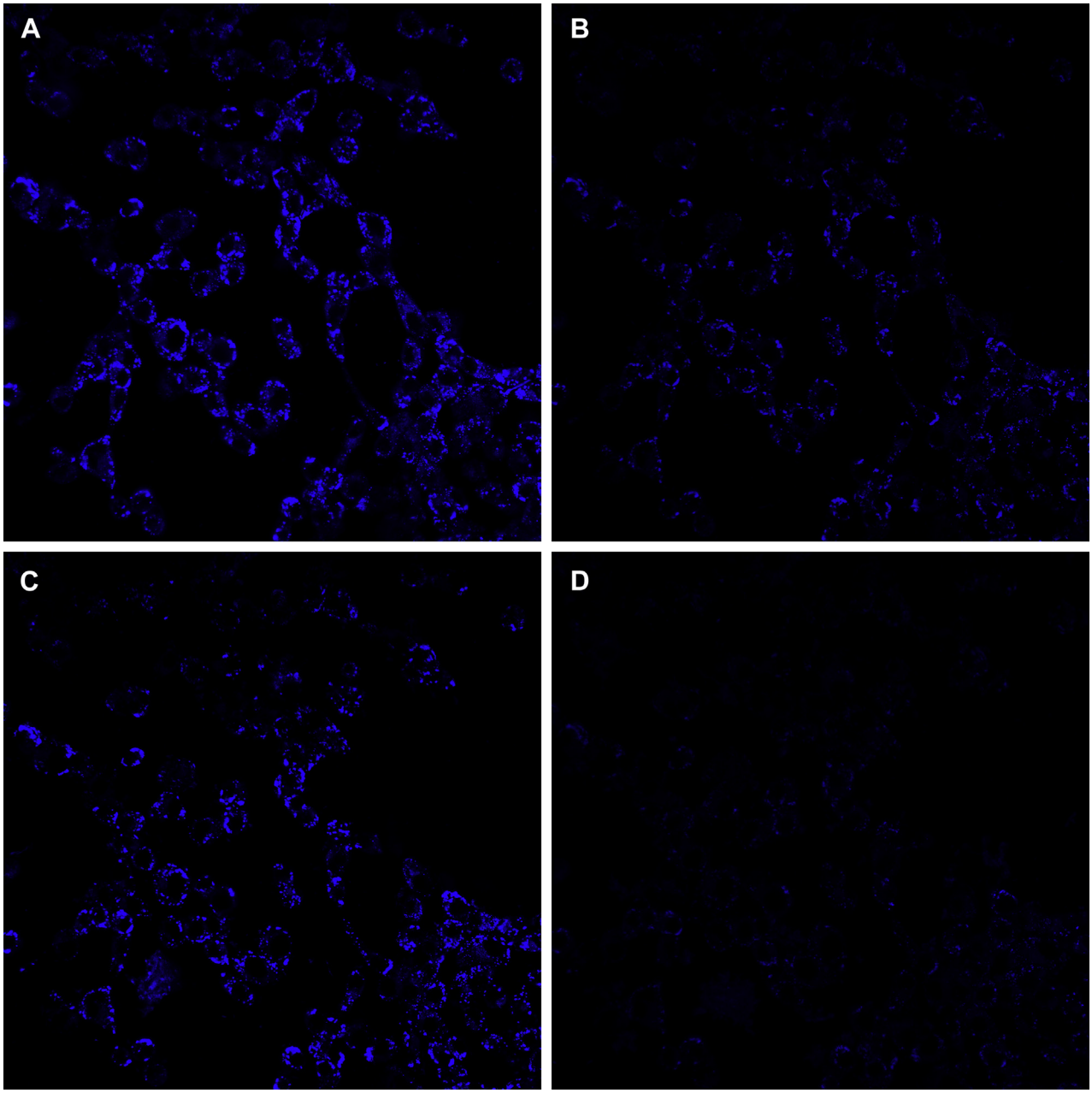

FIGURE 1. Laser confocal microscope images of glutathione probe in cal-27 cells (magnification ×20). *A*, Fluorescence image of cal-27 cells incubated with glutathione probe (10 μmol/L) for 20 minutes. *B*, Fluorescence image of cal-27 cells treated with 100-μmol/L hydrogen peroxide for 30 minutes. *C*, Fluorescence image of cal-27 cells treated with 1-mmol/L *N*-acetyl-L-cysteine for 1 hour. *D*, Fluorescence image of cal-27 cells treated with 50-μmol/L ethacrynic acid for another 30 minutes.

Zhou et al. Glutathione in Oral Squamous Cell Carcinoma. J Oral Maxillofac Surg 2017.

was observed using an excitation wavelength of 340 nm (Fig 1A). After 100-μmol/L H_2O_2 was added to the cells for 30 minutes, a decrease in the blue fluorescence could be observed (Fig 1B). Then the cells were treated with 1-mmol/L NAC (GSH precursor) for 1 hour, and the cal-27 cells emitted substantial blue fluorescence again (Fig 1C). Finally, after 50-μmol/L EA was added to the cells for another 30 minutes, as shown in Figure 1D, the blue fluorescence declined obviously to almost disappearance.

INTRACELLULAR ROS FORMATION WAS ENHANCED BY DEPLETION OF MTGSH

We applied the DCF-DA to assess intracellular ROS formation, and the green fluorescence was observed with the confocal microscope using an excitation wavelength of 500 nm. Before treatment, the cal-27 cells displayed intensive blue fluorescence and faint green fluorescence (Fig 2A), which indicated high levels of GSH and low levels of ROS. After the cells were treated with H_2O_2, green fluorescence was

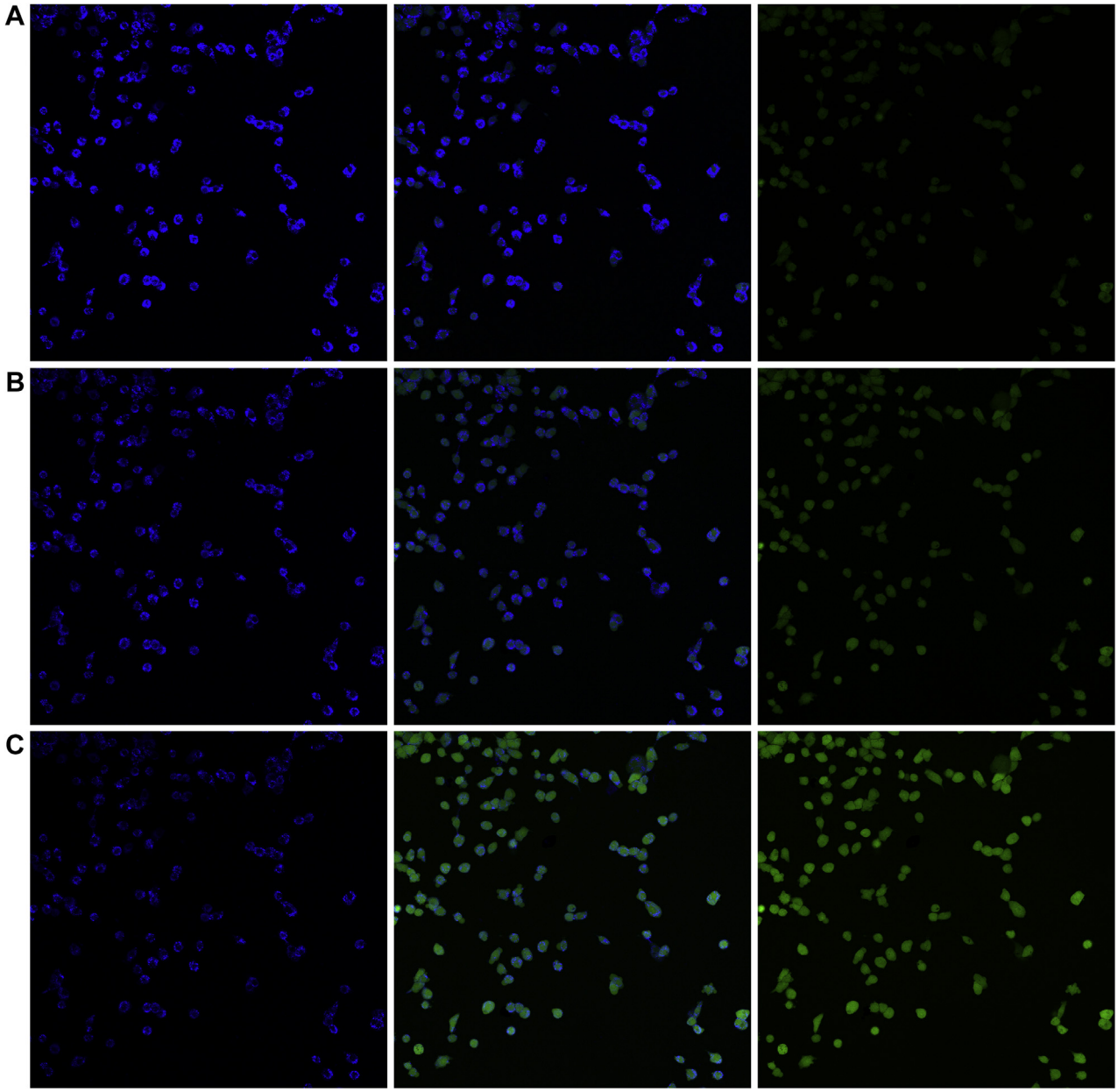

FIGURE 2. Laser confocal microscope images of glutathione and DCF-DA (2,7-dichlorofluorescin diacetate) probe in cal-27 cells (magnification ×10). *A*, Fluorescence images of cal-27 cells incubated with glutathione probe for 20 minutes and DCF-DA for 30 minutes. *B*, Fluorescence images of previous cells treated with 100-μmol/L hydrogen peroxide for 30 minutes. *C*, Fluorescence images of previous cells treated with 50-μmol/L ethacrynic acid for another 30 minutes.

Zhou et al. Glutathione in Oral Squamous Cell Carcinoma. J Oral Maxillofac Surg 2017.

enhanced and blue fluorescence was weakened (Fig 2B), which indicated ROS levels increased and GSH levels decreased. Especially after the cells were treated with EA, we could see that green fluorescence increased and blue fluorescence decreased more obviously compared with the effect of H_2O_2 (Fig 2C).

MITOCHONDRIAL ROS FORMATION WAS ENHANCED BY DEPLETION OF MTGSH

Mitotracker Red CM-H_2XRos can be concentrated and retained in mitochondria when apoptosis occurs. We applied Mitotracker Red CM-H_2XRos, which emitted intense red fluorescence at the excitation wavelength of 579 nm to locate mitochondria. Before treatment, the cal-27 cells displayed intensive blue fluorescence and faint green fluorescence as described earlier. The merging of mitochondria and GSH displayed intense purple fluorescence, which indicated high mtGSH levels, whereas the merged image of mitochondria and ROS displayed feeble orange fluorescence, which indicated low mitochondrial ROS levels (Fig 3A). After the cells were treated with 100-μmol/L H_2O_2 for 30 minutes, the blue fluorescence attenuated and the green fluorescence intensified. Accordingly, the merged purple fluorescence attenuated and the merged orange fluorescence intensified, which indicated that mtGSH levels decreased and mitochondrial ROS levels increased (Fig 3B). Then, after 50-μmol/L EA was added to the cells for the next 30 minutes, a substantial decrease in blue emission and a substantial increase in green emission could be viewed, and the merged purple fluorescence attenuated and the merged orange fluorescence intensified obviously (Fig 3C).

EVERSION OF PHOSPHATIDYLSERINE WAS ENHANCED BY DEPLETION OF MTGSH

Intracellular ROS formation and depletion of GSH are associated with apoptosis.[24,25] So we tried to apply the GSH selective probe to show the correlation between depletion of mtGSH and apoptosis. The annexin V–FITC probe displayed green fluorescence at an excitation wavelength of 488 nm. Before treatment, the annexin V–FITC probe in the cal-27 cells displayed feeble green fluorescence and the GSH probe emitted intense blue fluorescence (Fig 4A), which proved that high levels of GSH could prevent cell apoptosis.[26,27] When 100-μmol/L H_2O_2 was added for 30 minutes along with the cells, blue fluorescence attenuated and green fluorescence intensified (Fig 4B). Subsequently, after the cal-27 cells

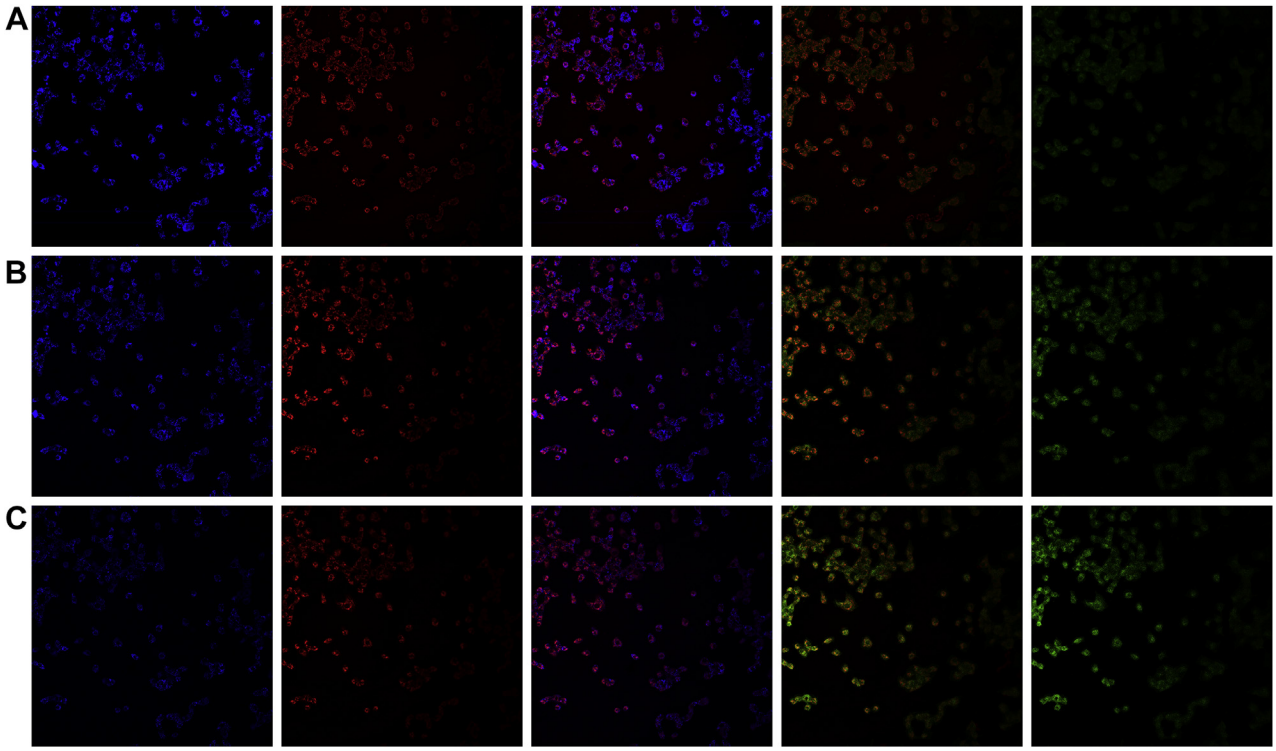

FIGURE 3. Laser confocal microscope images of glutathione, DCF-DA (2,7-dichlorofluorescin diacetate), and Mitotracker Red CM-H_2XRos probe in cal-27 cells (magnification ×10). *A*, Fluorescence images of cal-27 cells incubated with glutathione probe for 20 minutes, DCF-DA for 30 minutes, and Mitotracker Red CM-H_2XRos for 20 minutes. *B*, Fluorescence images of previous cells treated with 100-μmol/L hydrogen peroxide for 30 minutes. *C*, Fluorescence images of previous cells treated with 50-μmol/L ethacrynic acid for another 30 minutes.

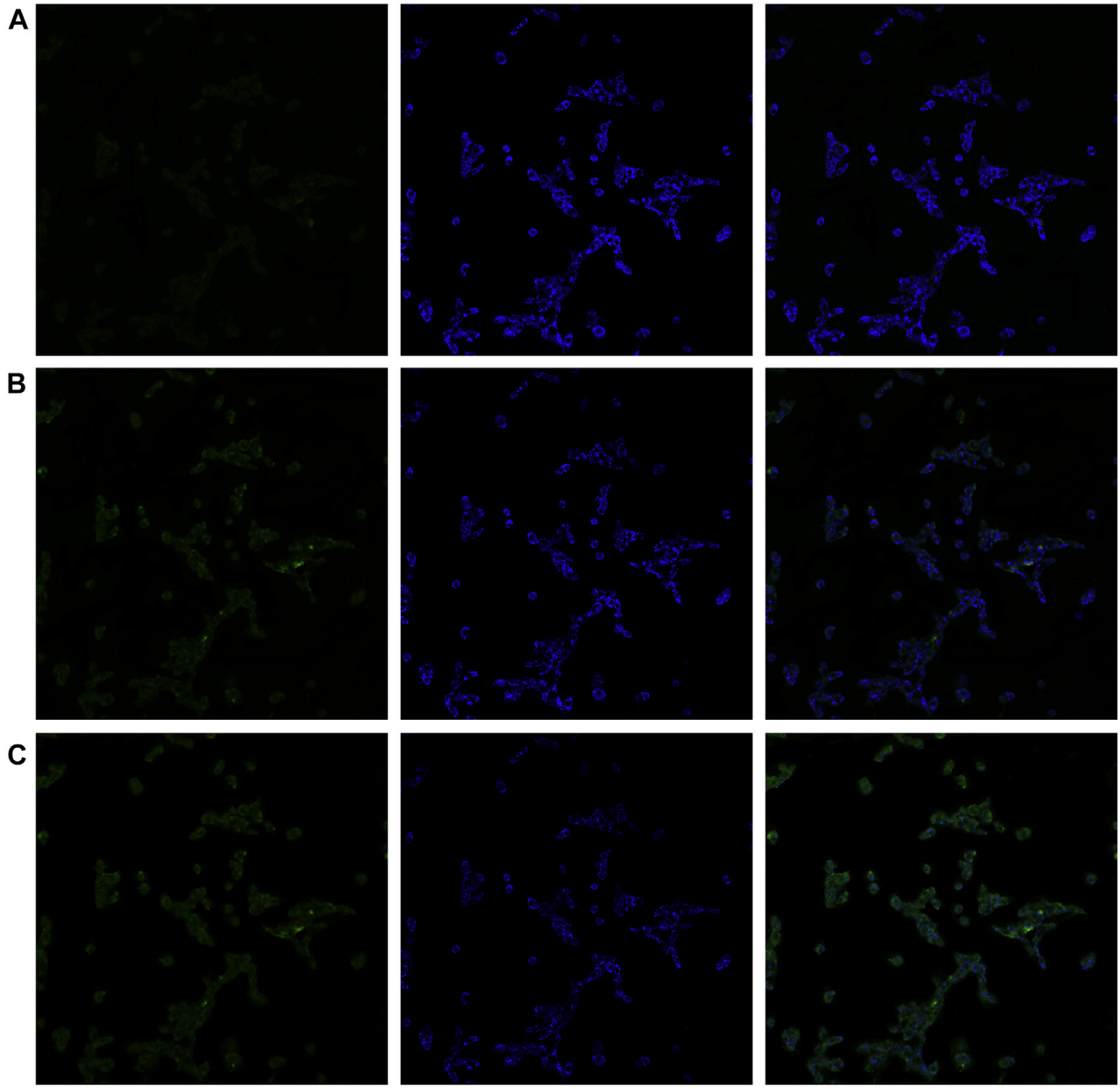

FIGURE 4. Laser confocal microscope images of glutathione and annexin V–fluorescein isothiocyanate probe in cal-27 cells (magnification ×10). *A*, Fluorescence images of cal-27 cells incubated with glutathione probe for 20 minutes and annexin V–fluorescein isothiocyanate probe for 10 minutes. *B*, Fluorescence images of previous cells treated with 100-μmol/L hydrogen peroxide for 30 minutes. *C*, Fluorescence images of previous cells treated with 50-μmol/L ethacrynic acid for another 30 minutes.

Zhou et al. Glutathione in Oral Squamous Cell Carcinoma. J Oral Maxillofac Surg 2017.

were treated with 50-μmol/L EA for the next 30 minutes, blue fluorescence nearly disappeared and the cells gave rise to a substantial increase in green emission (Fig 4C).

LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL WAS ENHANCED BY DEPLETION OF MTGSH

The decline of mitochondrial membrane potential ($\Delta\Psi m$) is an early landmark event of apoptosis.[28] In our study, we used the JC-1 probe to examine loss of mitochondrial membrane potential of the cal-27 cells after oxidative stress and depletion of mtGSH. Before treatment, high levels of GSH in the cal-27 cells emitted intense blue fluorescence. At this time, the JC-1 probe mainly existed in the form of polymer and emitted intense red fluorescence (Fig 5A). Figures 5B and C show that after the cal-27 cells were treated with H_2O_2 and EA consecutively, the blue fluorescence weakened rapidly, which

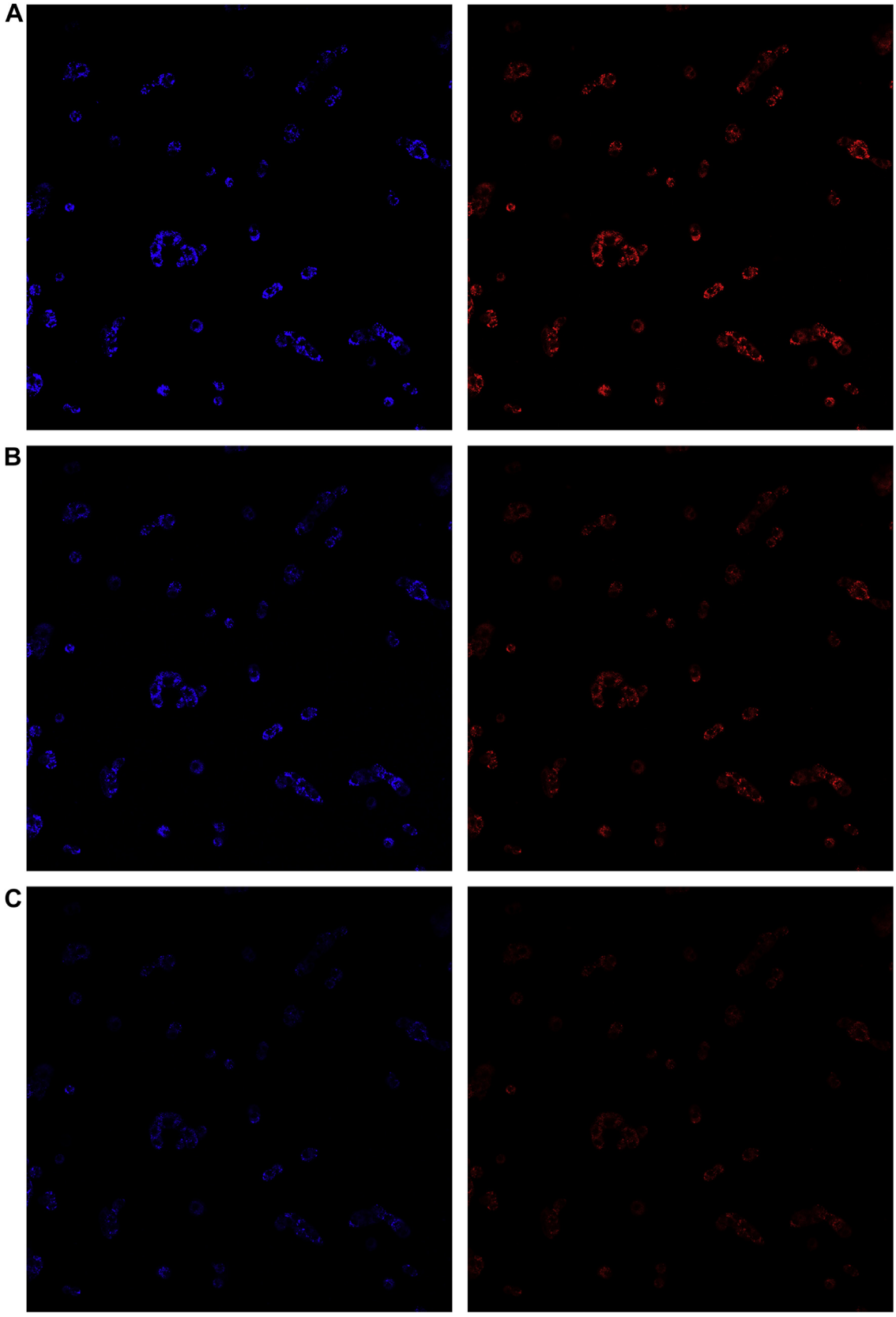

FIGURE 5. Laser confocal microscope images of glutathione and JC-1 probe in cal-27 cells (magnification ×10). *A*, Fluorescence images of cal-27 cells incubated with glutathione probe for 20 minutes and JC-1 polymer probe for 20 minutes. *B*, Fluorescence images of previous cells treated with 100-μmol/L hydrogen peroxide for 30 minutes. *C*, Fluorescence images of previous cells treated with 50-μmol/L ethacrynic acid for another 30 minutes.

Zhou et al. Glutathione in Oral Squamous Cell Carcinoma. J Oral Maxillofac Surg 2017.

indicated intracellular GSH levels decreased. At the same time, loss of mitochondrial membrane potential led to decomposition of the JC-1 polymer and a substantial decrease in red emission in the cal-27 cells.

Discussion

The main problem confronted in the treatment of OSCCs is resistance to chemotherapy and radiotherapy with the mechanism of ROS formation.[14] However, GSH is a substantial factor that correlates with resistance to chemotherapy agents such as cisplatin.[29] Recent studies have proved that the breaking of the cellular redox equilibrium is critical to the progression of apoptosis, which can be prevented by the prohibition of GSH depletion. Thus, the depletion of GSH is thought to be a promising strategy of decreasing chemotherapy resistance and inducing apoptosis through both extrinsic and intrinsic apoptotic pathways.[8] GSH is synthesized in cytoplasm and then distributed into several organelles including mitochondria. Although possessing only about 10 to 15% of the total cell, the concentration of mtGSH is higher than other compartments because of the tiny volume of mitochondria.[13] Moreover, mitochondria are the main source of ROS and depend highly on mtGSH to deal with ROS formation and prevent oxidative stress. Otherwise, oxidative stress can cause dysregulation of mitochondria to induce the apoptotic cascade.[30] Therefore, mtGSH is important in protecting tumor cells from chemotherapy agents.[10] Mitochondrial GSH can not only prevent accumulation of ROS but also protect mitochondria from oxidative damage.[8,31] Direct depletion of mtGSH can induce or facilitate apoptosis even if without enough oxidative stress.[16] Oxidative stress and a few GSH depletion agents, such as buthionine sulfoximine, just decrease GSH of cytoplasm, but not GSH in mitochondria.[12] Therefore, EA—a specific inhibitor of mtGSH—was applied to enhance oxidative stress and apoptosis of cal-27 cells induced by exogenous H_2O_2.

In our study, after cal-27 cells were incubated with the GSH fluorescent probe for 20 minutes, intense blue fluorescence positively correlating with GSH levels could be observed. Then the cells were treated with exogenous H_2O_2 and EA, which act as chemotherapy agents for different tests. Initially, a substantial decrease in blue fluorescence, which meant a rapid decrease in the cellular GSH level, could be observed, proving that the probe could detect GSH effectively and instantaneously. DCF-DA and Mitotracker Red CM-H_2XRos probes were then used to detect both intracellular and mitochondrial ROS formation and investigate whether cellular oxidative stress occurred. The results showed that along with the rapid decrease in GSH levels, intracellular ROS was formed after cells were treated with exogenous H_2O_2 and ROS formation was enhanced by the depletion of mtGSH. Mitochondrial ROS also was formed when the cells were treated with exogenous H_2O_2, and the formation of mitochondrial ROS was enhanced by EA; these findings prove that the depletion of GSH by H_2O_2 could induce cellular and mitochondrial oxidative stress, which was enhanced by EA, the scavenger of mtGSH. ROS formation and depletion of GSH were associated with apoptosis.[24,25] Eversion of phosphatidylserine meant pristine apoptosis. The decline of mitochondrial membrane potential is an early landmark event of apoptosis and usually implies that apoptosis is irreversible.[6,28-31] Depletion of cellular GSH is a hallmark during earlier stages of apoptosis and can induce cells to be susceptible to an apoptotic stimulus or directly induce cells to undergo apoptosis by either opening the permeability transition pore or activating caspases.[8] Further efforts were made to find whether oxidative stress would induce apoptosis of OSCCs. Annexin V–FITC and JC-1 probes were to detect the eversion of phosphatidylserine and the loss of the transmembrane potential of mitochondria. As we expected, apoptosis occurred after the depletion of GSH and was enhanced by EA. As the result of this research, the course of oxidative stress and the apoptosis, along with the rapid decrease in cellular GSH, could be visualized by the GSH selective fluorescent probe.

There are already a few fluorescent probes that have been devised to detect intracellular GSH levels. However, to our knowledge, most of these probes were simply applied to show alteration of GSH levels of HeLa cells or living animals.[18,32] In our study, we used a novel fluorescent probe[19] to detect alteration of GSH levels in living oral squamous cell carcinoma cells during the course of oxidative stress and apoptosis induced by exogenous H_2O_2 and EA for the first time. The occurrence of oxidative stress and apoptosis of the OSCCs could be visualized. Through variation of fluorescence intensity, we could make clear that depletion of mtGSH with EA could enhance oxidative stress and apoptosis. The relationship between decrease in cellular GSH and apoptosis of OSCCs could be visualized directly. Variation of fluorescence intensity, which represented intracellular alteration of GSH levels, cellular ROS formation, mitochondrial ROS formation, and apoptosis occurrence, respectively, could be visualized under laser confocal fluorescence microscopy. All these cells were treated and observed consecutively, so we could visualize the alteration of the fluorescence under the same vision.

The effect of this study indicated that the fluorescent probe could detect cellular GSH effectively

and depletion of mtGSH with EA, which enhanced oxidative stress and apoptosis of OSCCs induced by exogenous H_2O_2. In our study, we assumed the role of exogenous H_2O_2 as a chemotherapeutic drug. So we thought depletion of mtGSH was able to enhance oxidative stress and apoptosis of OSCCs during chemotherapy. However, this study lacked a quantitative analysis and more experimental research is needed to apply the probes in clinical trials.

Our study showed that the GSH selective fluorescent probe can be applied to detect alteration of cellular GSH levels sensitively during the course of oxidative stress and apoptosis of OSCCs induced by exogenous H_2O_2 and enhanced by depletion of mtGSH. The greatest advantage of the GSH probe is that we can visualize the intact process in real time and quantitatively. At last, we can draw the conclusion that depletion of mtGSH could enhance apoptosis of the cal-27 cells induced by oxidative stress.

Acknowledgment

Our laser confocal scanning imaging work (Zeiss LSM 780; Carl Zeiss, Germany) was performed at the Microscopy Characterization Facility, Shandong University, Jinan, China.

References

1. Nakaoka T, Ota A, Ono T, et al: Combined arsenic trioxide-cisplatin treatment enhances apoptosis in oral squamous cell carcinoma cells. Cell Oncol 37:119, 2014
2. Toyokuni S: Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 49:91, 1999
3. Block KI, Koch AC, Mead MN, et al: Impact of antioxidant supplementation on chemotherapeutic toxicity: A systematic review of the evidence from randomized controlled trials. Int J Cancer 123:1227, 2008
4. Lecane PS, Karaman MW, Sirisawad M, et al: Motexafin gadolinium and zinc induce oxidative stress responses and apoptosis in B-cell lymphoma lines. Cancer Res 65:11676, 2005
5. Poyton RO, Ball KA, Castello PR: Mitochondrial generation of free radicals and hypoxic signaling. Trends Endocrinol Metab 20:332, 2009
6. Sen CK, Packer L: Antioxidant and redox regulation of gene transcription. FASEB J 10:709, 1996
7. Singh S, Khan AR, Gupta AK: Role of glutathione in cancer pathophysiology and therapeutic interventions. J Exp Ther Oncol 9: 303, 2012
8. Franco R, Cidlowski JA: Apoptosis and glutathione: Beyond an antioxidant. Cell Death Differ 16:1303, 2009
9. Lushchak VI: Glutathione homeostasis and functions: Potential targets for medical interventions. J Amino Acids 2012:736837, 2012
10. Landriscina M, Maddalena F, Laudiero G, et al: Adaptation to oxidative stress, chemoresistance and cell survival. Antioxid Redox Signal 11:2701, 2009
11. Traverso N, Ricciarelli R, Nitti M, et al: Role of glutathione in cancer progression and chemoresistance. Oxid Med Cell Longev 2013:972913, 2013
12. Estrela JM, Ortega A, Obrador E: Glutathione in cancer biology and therapy. Crit Rev Clin Lab Sci 43:143, 2006
13. Marí M, Morales A, Colell A, et al: Mitochondrial glutathione, a key survival antioxidant. Antioxid Redox Signal 11:2685, 2009
14. Engel RH, Evens AM: Oxidative stress and apoptosis: A new treatment paradigm in cancer. Front Biosci 11:300, 2006
15. Hockenbery DM, Giedt CD, O'Neill JW, et al: Mitochondria and apoptosis: New therapeutic targets. Adv Cancer Res 85:203, 2002
16. Ortega AL, Mena S, Estrela JM: Glutathione in cancer cell death. Cancers 3:1285, 2011
17. Muyderman H, Wadey AL, Nilsson M, Sims NR: Mitochondrial glutathione protects against cell death induced by oxidative and nitrative stress in astrocytes. J Neurochem 102:1369, 2007
18. Zhai D, Lee SC, Yun SW, et al: A ratiometric fluorescent dye for the detection of glutathione in live cells and liver cancer tissue. Chem Commun 49:7207, 2013
19. Zhu B, Zhang X, Li Y, et al: A colorimetric and ratiometric fluorescent probe for thiols and its bioimaging applications. Chem Commun 46:5710, 2010
20. Park WH: Anti-apoptotic effect of caspase inhibitors on H_2O_2-treated HeLa cells through early suppression of its oxidative stress. Oncol Rep 31:2413, 2014
21. Ploemen JH, Van Schanke A, Van Ommen B, et al: Reversible conjugation of ethacrynic acid with glutathione and human glutathione S-transferase P1-1. Cancer Res 54:915, 1994
22. Muyderman H, Nilsson M, Sims NR: Highly selective and prolonged depletion of mitochondrial glutathione in astrocytes markedly increases sensitivity to peroxynitrite. J Neurosci 24: 8019, 2004
23. Wang R, Li C, Song D, et al: Ethacrynic acid butyl-ester induces apoptosis in leukemia cells through a hydrogen peroxide-mediated pathway independent of glutathione S-transferase p1-1 inhibition. Cancer Res 67:7856, 2007
24. Zuo L, Motherwell MS: The impact of reactive oxygen species and genetic mitochondrial mutations in Parkinson's disease. Gene 532:18, 2013
25. Circu ML, Aw TY: Glutathione and modulation of cell apoptosis. Biochim Biophys Acta 1823:1767, 2012
26. Friesen C, Kiess Y, Debatin KM: A critical role of glutathione in determining apoptosis sensitivity and resistance in leukemia cells. Cell Death Differ 11(Suppl 1):S73, 2004
27. Cazanave S, Berson A, Haouzi D, et al: High hepatic glutathione stores alleviate fas-induced apoptosis in mice. J Hepatol 46:858, 2007
28. Hong J, Samudio I, Chintharlapalli S, et al: 1,1-Bis (3'-indolyl)-1-(p-substituted phenyl) methanes decrease mitochondrial membrane potential and induce apoptosis in endometrial and other cancer cell lines. Mol Carcinog 47:492, 2008
29. Köberle B, Tomicic MT, Usanova S, et al: Cisplatin resistance: Preclinical findings and clinical implications. Biochim Biophys Acta 1806:172, 2010
30. Manoli L, Alesci S, Blackman MR, et al: Mitochondria as key components of the stress response. Trends Endocrinol Metab 18: 190, 2007
31. Hollins DL, Suliman HB, Piantadosi CA, et al: Glutathione regulates susceptibility to oxidant-induced mitochondrial DNA damage in human lymphocytes. Free Radic Biol Med 40:1220, 2006
32. Yin J, Kwon Y, Kim D, et al: A cyanine based fluorescence probe for highly selective detection of glutathione in cell cultures and live mice tissues. J Am Chem Soc 136:5351, 2014